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PRINCIPAL INVESTIGATOR: Michael F. Press, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California  
Los Angeles, CA 90033

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*Michael J. Jones* 8/29/97  
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**INTRODUCTION:** This is a molecular epidemiologic case-control study of breast carcinoma *in situ* in Los Angeles County designed to address issues related to the cause and progression of breast CIS by determining epidemiologic risk factors, characterizing selected molecular genetic alterations and prospectively assessing disease progression.

Breast carcinoma *in situ* (CIS) is a significant public health problem in Los Angeles County and throughout the United States. Incidence of this disease has been rising steadily, with little epidemiologic or biologic information about breast CIS available. In Los Angeles county, incidence of breast CIS increased from approximately 4 cases per 100,000 women in 1980 to 15 cases per 100,000 women in 1992.

The specific aims of the research are 1.) to assess epidemiologic risk factors associated with development of breast CIS, 2.) to determine how frequently specific oncogenes or the p53 tumor suppressor gene are altered in breast CIS, 3.) to investigate potential relationships between various epidemiologic risk factors and somatic genetic alterations and 4.) to assess long-term the association of these factors with disease progression.

During the four-year grant period we plan to interview approximately 100 black women and 426 white women (including Hispanics) aged 35-64 years who are diagnosed with breast CIS and who are residents of Los Angeles County, are US-born, and English speaking. The study will utilize 490 black and 490 white control subjects selected by random digit dialing in Los Angeles County who will have been interviewed as part of the Women's CARE Study, a multicentered case-control study of invasive breast cancer being conducted concurrently with this proposed study. We will obtain paraffin-embedded tumor tissue from the pathology laboratories where the patients were diagnosed. This case-control study will evaluate reproductive history (early menarche, late menopause, nulliparity or late first birth), lack of participation in physical activity/exercise, positive family history, race (white vs. black), high body mass and exposure to exogenous hormones (oral contraceptives and estrogen or combined estrogen/progestogen replacement therapy) for associations with an increased risk of developing breast CIS. Gene amplification in proto-oncogenes, known to be altered in invasive breast cancer, will be evaluated for changes in pre-invasive breast CIS. The HER-2/*neu* and PRAD1 oncogene will be evaluated for gene amplification by fluorescence *in situ* hybridization and for expression by immunohistochemistry. The p53 tumor suppressor gene, known to be mutated and/or overexpressed in approximately 30% of invasive breast cancers, will be characterized for p53 mutations and overexpression by DNA sequencing and immunohistochemistry. We will determine if any genetic mutations are associated with any of the epidemiologic risk factors investigated. We will also determine if any of the genetic alterations or epidemiologic characteristics are associated with an increased risk of recurrence or progression to invasive disease.

## **PROGRESS DURING YEAR 01.**

## **EXPERIMENTAL METHODS AND PROCEDURES.**

### **1. Epidemiologic Study Methods.**

We are conducting a population-based, case-control study of breast CIS in Los Angeles County using the population-based Cancer Surveillance Program (CSP) at the University of Southern California to identify newly diagnosed patients. Dr. Leslie Bernstein, Co-Principal Investigator, also serves as Scientific Director of the CSP. This investigation is expected to be the largest and most comprehensive epidemiologic and the largest molecular biological characterization of breast CIS yet undertaken. This study will utilize control subjects and methodology from the Women's Contraceptive and Reproductive Experiences (CARE) Study described below.

Timing of the conduct of this proposed study is critical in terms of interviewing women newly diagnosed with breast CIS in Los Angeles County who are eligible for the study. As described below,

we are conducting a case-control study of invasive breast cancer, funded by NICHD, the Women's CARE Study. The Women's CARE Study provide the controls for this epidemiologic study of breast CIS. The breast CIS cases and the Women's CARE Study controls are interviewed concurrently in order to be valid. To do this case-control study of breast CIS independently of the Women's CARE Study would nearly double the costs of the epidemiologic component of our budget as we would need to develop a control identification mechanism, and recruit and interview controls.

Women's Contraceptive and Reproductive Experiences (CARE) Study. The Women's CARE Study is a multicentered case-control study of invasive breast cancer being conducted among women aged 35-64 years in five areas of the US including Los Angeles (PI: Leslie Bernstein, Ph.D.). The Los Angeles Field Center will obtain interviews with 1200 patients with invasive breast cancer and 1200 controls. Half of the patients and half of the controls will be black women; the remainder will be white women (including Hispanic women). Since this study is being conducted concurrently with the study of breast CIS, we utilize the questionnaire that has been designed for the Women's CARE Study and take advantage of the control subjects being interviewed for the Women's CARE Study.

Subject eligibility for the Women's CARE Study is limited to women aged 35-64 years of age who were born in the United States, who are fluent in English, and who have no prior diagnosis of breast CIS or invasive breast cancer.

Controls are selected for the Women's CARE Study by random digit dialing methods. The Centers for Disease Control (CDC) which serves as the Data Coordinating Center for the study has a subcontract with WESTAT for this purpose. Controls are being frequency matched by race and 5-year age category to the case population in Los Angeles. In evaluating the incidence of breast CIS in black and white women in Los Angeles County in 1991 and 1992, we note that the frequency counts in each age group are relatively constant. Since the number of white cases in the Women's CARE Study in each 5-year age category is being held constant in Los Angeles by randomly selecting different proportions in each age group, the distribution of white controls should be optimum for the breast CIS study. For black women, the Women's CARE Study distribution is more heavily weighted toward the older age groups, but because of the fact that 490 controls will be available for the 100 breast CIS cases (see next section below), sufficient controls will be available in each 5-year age category.

The CDC has provided a study management software system (CARETRAC, programmed in Foxpro) that will be utilized for the breast CIS study. This study management system includes automatic generation of letters to physicians and subjects after subject information is entered. It records dates of each step in the process of subject recruitment and has substantial report writing capabilities. It allows for the designation of selection fractions for each 5-year age and race group of women so that one can randomly select patients from among eligible subjects. This selection is done at the time that all eligibility criteria for a given subject have been entered into CARETRAC. CARETRAC maintains records of all contacts with physicians, patients and potential controls.

The Women's CARE Study has implemented strict quality control and data management procedures, all of which are incorporated into this study of breast CIS. These procedures include the selection of a 10% random sample of interviewed subjects for re-interview by the study supervisor. Each questionnaire is visually edited by the interviewer and by the study supervisor. Questionnaire data are entered into the computer using data entry software, SURVEY, provided by the CDC which has programmed checks for out-of-range values and correct skip patterns.

Recruitment of Breast CIS Patients. The selection of cases are restricted to all female cases of breast CIS who are consecutively identified by the CSP and satisfy the following criteria:

1. Age: 35-64.
2. Race: black or white.
3. Birthplace: United States.
4. Language: English speaking.
5. Diagnosis: Breast carcinoma *in situ* (ductal or lobular)
6. Date of Diagnosis: July 1, 1996 and June 30, 1999.
7. Restrictions: No prior diagnosis of breast CIS or invasive breast cancer.



The CSP, the population-based cancer registry for Los Angeles County is operated by the Department of Preventive Medicine at the University of Southern California School of Medicine. As noted above the Co-PI of this study, Dr. Bernstein, serves as the Scientific Director for the CSP. The CSP began cancer surveillance in Los Angeles County in 1970 and has had nearly complete ascertainment of all incident cancers diagnosed among Los Angeles County residents since 1972. From 1970 until June, 1987, the CSP utilized an active case ascertainment system for identification of cancer patients by deploying medical records technicians to all hospital pathology facilities to search for any reports of cancer. This system was modified somewhat in mid-1987 when the CSP was designated as one of the 10 regional cancer registries for the California Cancer Registry, the statewide registry. At this time state legislation placed the burden of reporting cancer onto the facility or physician's practice where the diagnosis of cancer was made. The CSP has maintained its active case ascertainment component to insure complete reporting and to be able to maintain its repository of pathology reports on all histologically confirmed incident cancers diagnosed among Los Angeles County residents since 1972. In 1992, the CSP was designated as one of the Surveillance, Epidemiology, and End Results (SEER) registries. As a SEER registry, the CSP has instituted follow-up activities on all incident cases diagnosed from 1992 onward to maintain up-to-date information on vital status.

We expect to identify 125 African-American women newly diagnosed with a first primary breast CIS in the three year accrual period and, based on our current response rates in the Women's CARE Study, to interview 100 (80%) of these women. Nearly all of the 125 women will be US born.

We expect to identify 354 white women newly diagnosed with a first primary breast CIS each year who are in the given age range and who are born in the US. We will randomly select half of these women (N = 177 annually) for interview and expect to successfully complete interviews with 142 annually (80%). The random selection process is part of the CARETRAC System currently utilized for the Women's CARE Study and is based on a well-characterized random number generator. We will accrue white women with breast CIS for 3 years and expect to interview a total of 426 white patients in addition to the 100 African-American patients.

Cases for case-control studies are identified by the CSP using a Rapid Case Ascertainment (RCA) procedure. Medical records technicians at the CSP currently visit all pathology laboratories in Los Angeles County on a frequent regular basis, with frequency based on the total number of cancer patients diagnosed at the facility. Each facility is visited at least once a month. The technicians obtain the pathology reports documenting the breast CIS diagnosis, obtain demographic information on the patient (for determination of study eligibility) and express mail or hand deliver the reports to the CSP the same week that the patient is identified. These are then delivered to the Women's CARE Study office across the street from the CSP. Based on this procedure, we expect to identify and interview nearly all breast CIS patients within 3 months of their initial diagnosis.

We utilize the procedures currently used in the Women's CARE Study to obtain physician permission to contact a patient. Currently, we telephone physicians for permission to contact their patients and to obtain updated information regarding the patient's address and any contraindications to contacting the patient. After physician permission is granted, we make initial contact with the patient by letter. This letter advises the patient of the purpose and nature of the study and of our intent to contact her to arrange a personal interview. The patient is then contacted by telephone to seek cooperation and to arrange an appointment for the personal interview. The control contact procedures are identical to those for the case, after the physician has granted us permission to contact the patient.

Batches of eligible controls for the Women's CARE Study are provided to Dr. Bernstein every two weeks by WESTAT following their identification by random digit dialing methods. These methods use a sampling frame of Los Angeles County telephone exchanges that is updated at least every six months.

Data Acquisition. At the time of the interview, informed consent is obtained for the interview, and for patients, authorization to receive biopsy specimen tissue blocks. The epidemiologic interview requires approximately 75 minutes to administer. The questionnaire (see Appendix) contains questions on established and suspected breast cancer risk factors, addressing demographics, pregnancies, menstruation, menopause, surgeries of the breast, ovaries and uterus, hormonal contraception, hormone

replacement, other medications, infertility, medical history, mammogram history, physical activity, body size at various ages, cigarette and alcohol consumption histories, first and second degree maternal and paternal family history of cancer and prenatal exposures. The interviewer utilizes a calendar of life events on which she and the participant record important events, and reproductive and contraceptive history. A photograph album of exogenous hormone preparations is also used to identify specific formulations a woman has used.

All interviewers for the Women's CARE Study undergo a rigorous training period and accreditation by CDC and WESTAT staff before entering the field for the Women's CARE Study. In order to insure that the breast CIS interviewer interviews both breast CIS cases and controls, and to insure that we do not have an unequal balance of patient and control interviews in the Women's CARE Study, the interviewer supported by this study is integrated into the interviewing staff of the Women's CARE Study. The Women's CARE Study currently employs 2.6 FTE interviewers. With the interviewer for the breast CIS patients there are 3.6 FTE interviewers and they are assigned equally to the tasks of interviewing invasive and *in situ* cases as well as controls. This ensures that no bias enters into the assignment for either study.

**Retrieving Tissue Specimens:** The Women's CARE Study and the CSP have in place mechanisms for the accrual of tissue blocks from surgical pathology laboratories where the biopsy diagnosis was reported since Dr. Bernstein and Dr. Press are collaborating on two other studies including the Women's CARE Study which examines the relationship of prognostic factors and genetic alterations to invasive breast cancer risk factors. Briefly, a request is made by the study staff to the Pathology Retrieval Resource at the CSP for the particular specimens required. This request is accompanied by the patient's signed release of the materials requested. A processing fee is paid to cover the costs of retrieving the materials from the originating laboratory. Tissue specimens are logged into the study database and forwarded to Dr. Press's laboratory for processing.

**Data Processing:** As noted above, after the questionnaire has been edited by the interviewer and study supervisor, it is key entered into SURVEY, a software package that includes data edits. Any inconsistencies in the data are corrected as they are identified. If necessary, respondents are recontacted to clarify information.

**Statistical Analyses:** For the case-control study comparisons, we will use standard methods of statistical analysis for unmatched studies. Our approach will be to set up a series of 2 by 2 tables for dichotomous variables and of k by 2 tables for variables with k response levels in the preliminary analyses. Odds ratios and corresponding tests for significance and tests for trends will be computed. We will then utilize unconditional logistic regression to examine multivariate models, evaluating possible confounding and effect modification. All analyses will include adjustment for age in the model. In addition to assessing the magnitude of risk associated with epidemiologic risk factors for breast cancer, we will pay particular attention to whether specific molecular genetic alterations, such as *HER-2/neu* or p53 overexpression modify the effects of these factors on risk of breast CIS. In addition, among breast CIS patients we will examine the associations of individual genetic markers with accepted risk factors for breast cancer (e.g., age, ages at menarche, first term pregnancy and menopause, family history of breast cancer) and other potential risk factors (e.g., physical activity, alcohol consumption). These analyses will utilize standard methods for analysis of categorical data.

**Follow-up for Recurrence/Disease Progression:** Follow-up activities are performed on an annual basis following completion of the interview. For subsequent years after conclusion of the 4-year funding period, we plan to seek funds to continue the follow-up activities with a goal of completing a 10-year follow-up on these patients. Briefly, breast CIS patients will be actively followed by 1) maintaining contact with them annually to ascertain whether they have experienced any recurrence or progression of their disease, and if so, where treatment occurred and who was the treating physician; 2) maintaining contact with their current oncologist; and 3) by obtaining follow-up information annually from the tumor registry at the hospital at which they were originally diagnosed. Detailed information is obtained on dates of recurrences and diagnosis of more advanced disease. Pathology reports documenting these events is



obtained from the pathologist at the laboratories where these diagnoses are made. In addition, medical records are requested from the hospitals and physicians to document these events. As a SEER registry, the CSP maintains follow-up on all patients with regard to vital status, so that we can annually link our file of interviewed breast CIS patients with this database to determine if any patients have died.

## 2. Laboratory Methods.

**Histopathologic Assessment.** Tissue blocks and surgical pathology reports received for each of the cases are reviewed and coded. Microscopic tissue sections (4 microns thick), routinely stained with hematoxylin and eosin, from each tissue block are used for pathologic assessment of each CIS. The cases are classified by the principal investigator as ductal CIS (DCIS) or lobular CIS (LCIS). is sub classified as comedo, cribriform, papillary, micropapillary, solid, or mixed. The presence or absence of other breast pathology including especially ductal hyperplasia and atypical hyperplasia is recorded.

After review of sections from each of the tissue blocks available, the most representative tissue blocks is selected and a total of at least 15 additional sections are cut for the FISH analyses (HER-2/*neu* and cyclin D1), immunohistochemical analyses (ER, PR, HER-2/*neu* , and P53), single-strand conformation polymorphism (SSCP) (p53, exons 2-11) and DNA sequence analysis (p53 exons with mutations by SSCP).

**Assessment of Estrogen Receptor and Progesterone Receptor Content.** The presence or absence of estrogen receptor (ER) and progesterone receptor (PR) is determined with immunohistochemical assay of paraffin-embedded tissue sections. ER is demonstrated using a monoclonal ER antibody, 1D5, and PR is demonstrated using a monoclonal PR antibody, KD68, in the peroxidase anti-peroxidase technique as described (1). The ER and PR content is recorded as a percentage of positively immunostained cells. Breast CIS cases are characterized as receptor-positive if at least 10% of the CIS tumor cells contain receptor.

**Evaluation of Oncogene Expression.** Most studies of HER-2/*neu* oncogene expression in breast cancers have used immunohistochemistry as the primary or sole method of analysis. A variety of HER-2/*neu* antibodies have been used in these immunohistochemical studies, however, very little information has been available concerning the antibodies' ability to detect overexpression following tissue processing for paraffin-embedding. Therefore, we evaluated a series of antibodies, reported in the literature or commercially available, to assess their sensitivity and specificity as immunohistochemical reagents and have selected one of the most sensitive antibodies for our studies (2). Immunohistochemistry is used to determine oncogene expression levels in tissue sections. The HER-2/*neu* antibody is a rabbit polyclonal which we have described in previous publications (2, 3, 4, 5).

**IMMUNOHISTOCHEMISTRY.** Oncoprotein expression in breast CIS (as well as normal and hyperplastic breast epithelium) is assayed by immunohistochemistry using antibodies demonstrated to be sensitive in paraffin-embedded tissues. Our approach to immunohistochemistry involves screening a series of antibodies to the oncoprotein of interest with paraffin-embedded tissues having known expression levels for the oncoprotein. All available antibodies are systematically tested. The antibody with the best performance is selected for routine use in our assays. Our approach to assessing antibodies is described for HER-2/*neu* antibodies in a recent publication (2). This "screening" of antibodies for sensitivity in paraffin-embedded tissue is an important step. Most antibodies function well in frozen tissue, but only some are sensitive in paraffin-embedded tissue. This initial screening step ensures that the most sensitive and specific antibody is used for the assays. Antibodies are already available for each of the oncoproteins described in this proposal. The antibody to be used for HER-2/*neu* has already been selected (2) and results for p53 antibody selection are available.

The immunohistochemical staining method, described elsewhere in detail (2, 3, 4, 5), involves the sequential application of three antibodies to tissue sections as follows: 1.) primary rabbit (or mouse) anti-oncogene antibody, 2.) a secondary or bridging goat anti-rabbit (or -mouse) IgG antiserum (1:75 dilution; Sternberger Monoclonals, Inc.), and 3.) a rabbit (or mouse) peroxidase antiperoxidase antibody (1:75 dilution; Sternberger Monoclonals, Inc.). The primary antibody is incubated overnight at 4°C and

the secondary and tertiary antibodies are incubated at room temperature for half of an hour. After treatment with each antibody the tissue sections are washed with phosphate buffered saline. The immunoprecipitates are identified microscopically after incubation with the chromogen diaminobenzidine. Positive and negative test tissue sections are included with each immunohistochemical procedure as controls. Immunostaining of the various subcellular compartments is interpreted in each of the cell types. The amount of staining is scored in a blinded fashion as negative (no immunostaining), trace positive (weak membrane staining in minority of tumor cells), moderate immunostaining (distinct membrane staining in the majority of cells), or strong immunostaining (intense membrane staining in the majority of cells).

**Overexpression of p53 Tumor Suppressor Gene in Breast CIS.** p53 is a normal cellular protein which is important in regulating cell growth and metabolism, especially the G1-to-S transition of the cell cycle. However, p53 protein in normal cells of human tissues is present at a low level which is below the level identified by immunohistochemistry. Immunohistochemistry has proven to be a convenient way to distinguish normal expression (negative immunohistochemical staining) from p53 overexpression (positive nuclear immunostaining) in human breast cancers.

**IMMUNOHISTOCHEMISTRY.** Immunohistochemistry is a very useful method of assessing expression of tumor suppressor genes in tumor specimens. It permits identification of the cell type expressing the tumor suppressor and, if sensitive antibodies and techniques are used, relative expression levels on a cell-by-cell basis. Since immunohistochemistry can be used in paraffin-embedded as well as frozen tissue, it is a very versatile method that is well-suited to a study of breast CIS. We have already tested eleven different p53 antibodies (NCL-p53, CM-1 {Novocastra Labs}, DO-7 {DAKO Corp.}, BP53-12 {BioGenex}, Pab421, Pab1801, Pab 240, Pab246, Pab1620, DO-1 {Oncogene Science}, and CM-10 {Oncor, Inc.}) and selected the most sensitive and specific antibody in paraffin-embedded tissue (Chen and Press, manuscript in preparation). Each antibody has been tested both with and without antigen retrieval techniques (6).

**Analysis of p53 Mutations.** All of the breast CIS tissue samples are in paraffin-embedded tissue blocks necessitating use of genomic DNA for sequence analysis of p53. Tissue sections mounted on glass or plastic slides are examined microscopically, DCIS and LCIS (or atypical ductal hyperplasia or other histologic phenotype) are identified, and separated from one another by microdissection.

We have delayed DNA sequence analysis of the p53 gene from our breast CIS cases because we are testing a new DNA sequence technology in collaboration with a group at OncorMed, Inc. This technology is based on the use of microchips with assembled oligonucleotide sequences for hybridization of PCR amplified p53 from exons 2 - 11. We are testing 250 cancer specimens from our laboratory which have known p53 sequence results previously determined by single-strand conformation polymorphism (SSCP) screening and DNA sequence analysis. These specimens are being characterized the new DNA "chip" technology in a blinded fashion. The results of this approach will be compared with the results of SSCP and DNA sequence analysis to determine the sensitivity and specificity of this new technology and to use the discrepancies to further refine the DNA microchip for p53 analysis. If this technology can be used, we anticipate that this new method will greatly accelerate our ability to analyze p53. We also expect the new technology to be more sensitive for detection of all p53 mutations, since SSCP is variously reported to be able to detect from 70 to 95% of p53 mutations.

## RESULTS AND DISCUSSION.

**Epidemiologic Interviews.** Our interviews of women in Los Angeles County diagnosed with breast CIS is ahead of our projections in the grant application, partly because support from the SEER Registry Special Studies mechanism permitted us to begin interviewing prior to the start-up date of this USAMRMC grant. We have currently interviewed 283 women (122 of these within the last year) and have obtained blood samples from 221 study participants (22 are currently pending, 38 have refused and 2 are excluded because of anti-coagulant medications). Permission to study paraffin-embedded tissue blocks has been obtained from 258 study participants (5 are pending).

**Analysis of Breast CIS Tissue Blocks.** We have received paraffin-embedded tissue blocks from 69 participants and slides from an additional 32 participants for a total of 101 study participants. Tissue sections have been reviewed and immunohistochemical assays evaluated from 95 cases and 6 are currently pending. Histologic sections, either prepared from paraffin blocks or provided from referring laboratories, were reviewed from each case to confirm the diagnosis of breast CIS. CIS was not identified in routine hematoxylin-and-eosin stained histologic sections of seven cases. One case contained invasive breast carcinoma and was excluded from the study. In eight cases only hematoxylin-and-eosin stained histologic sections were provided; no unstained sections were provided for our subsequent analyses. The breast CIS cases were histologically subclassified as 17 comedo-CISs, 12 micropapillary CISs, 11 solid CISs, 8 cribriform CISs, 5 papillary CISs, 9 LCISs and 19 mixed CIS types. Additional sections were used for the assessment of estrogen receptor, progesterone receptor, HER-2/*neu* oncoprotein and p53 tumor suppressor in breast CIS.

**Estrogen Receptor.** The estrogen receptor (ER) content of 81 CIS cases was assessed by immunohistochemistry. 66 CIS cases were ER-positive (81%) and 15 were ER-poor (19%).

**Progesterone Receptor.** The progesterone receptor (PR) content of 81 CIS cases was assessed by immunohistochemistry. 57 CIS cases were PR-positive (70%) and 24 were PR-poor (30%).

**HER-2/*neu* Oncoprotein.** The HER-2/*neu* expression level of 81 CIS cases was assessed by immunohistochemistry. Overexpression of HER-2/*neu* oncoprotein was observed in 31 cases (38%) and low expression was observed in 50 cases (62%).

**p53 Tumor Suppressor Protein.** p53 tumor suppressor expression was evaluated in 81 cases by immunohistochemistry and 13 cases showed overexpression (16%) while 68 cases (84%) showed no overexpression.

**Cyclin D1.** Currently, cyclin D1 antibodies are being evaluated for sensitivity in paraffin-embedded tissue blocks. We will select one or two antibodies for immunohistochemical assessment of cyclin D1 protein product during the up-coming year.

**Analysis of p53 Gene for Mutations.** The p53 tumor suppressor gene is altered in more than half of human cancers. Although p53 mutations are common in invasive breast carcinoma, few have been identified in intraductal breast carcinomas (breast carcinoma *in situ*). During the initial part of the funding period, while patients were being interviewed, we pilot tested the methods for analysis of the p53 tumor suppressor gene that we were planning to use in the participant's tissue blocks. This permitted us to establish the methods for microdissection of breast carcinoma *in situ* and to demonstrate that p53 is mutated and overexpressed in intraductal breast carcinomas. The results of the pilot study of 40 breast carcinoma *in situ* cases is summarized below.

**Summary of findings in p53 pilot study.** The pilot study was undertaken to characterize p53 in a cohort of breast carcinoma *in situ* (CIS) cases, both with and without invasive disease, in order to confirm that p53 is mutated prior to invasion and to demonstrate that p53 mutations identified in intraductal carcinomas are related to p53 mutations found in invasive breast carcinomas. Among breast CISs without invasive disease, 22% had p53 mutations and 7% had DNA sequence alterations of unknown significance. p53 overexpression was identified in 37% of intraductal breast carcinomas. p53 mutations or alterations of unknown significance were concordant with p53 overexpression in 88% of breast CIS cases lacking invasive disease. Analysis of breast CIS with invasive disease demonstrated p53 mutations in 23% of cases and DNA alterations of unknown significance in 8%. Each carcinoma containing a p53 mutation in the breast CIS component had the identical mutation in the invasive component of the same tumor suggesting a clonal relationship between the two tumor components. p53 protein overexpression was identified in 38% of intraductal breast carcinomas with invasive disease and at approximately the same level in both components. Comparison of immunostaining and DNA sequence alterations showed a significant association between overexpression and mutations ( $p < 0.0001$ ). p53 mutations and p53 overexpression were relatively common in intraductal breast carcinomas but were not observed in adjacent normal breast lobules or ducts, indicating that p53 alterations usually occur prior to invasion of the breast carcinoma, as is observed for a number of other adult solid tumors. The findings have been submitted for publication (7) and a copy of the manuscript is included as an appendix.

***p53* Tumor Suppressor Gene.** In the past we have screened the *p53* gene for mutations using single-strand conformation polymorphism (SSCP) of each coding exon (exons 2 - 11). Those exons which demonstrate altered mobility are sequenced completely to identify the specific DNA sequence base change using either conventional dideoxy DNA sequence analysis or an automatic DNA sequencer. This approach for evaluation of *p53* gene mutations, although used by most investigators, is tedious and time-consuming. Because of the large number of cases to be evaluated in this study and because of the small size of the CIS tumor samples to be analyzed, we have begun to explore new methods for DNA sequence analysis which may prove to be faster and more efficient.

In collaboration with a group at OncorMed, Inc. we are currently testing high-density oligonucleotide DNA arrays for the analysis of *p53*. The use of high-density oligonucleotide DNA arrays, the so-called DNA chip technology, for DNA sequence analysis of known genes is an active area of investigation (8). Approximately 250 invasive cancers, previously characterized in my laboratory for *p53* mutations by SSCP screening of exons 2 - 11 and conventional DNA sequence analysis, are being evaluated in a blinded fashion using a prototype oligonucleotide DNA "*p53* chip" to assess the sensitivity and specificity of this technology for evaluation of *p53* mutations. Preliminary data from the first 70 cancers were unblinded this week during the preparation of this Progress Report and the results demonstrated that some *p53* mutations not identified by SSCP and conventional DNA sequence analysis were, nevertheless, identified with this prototype "*p53* chip". If the results of this approach prove to be successful for *p53* sequence analysis, then we will use this technology for analysis of *p53* in this USAMRMC study.

**Recommendations in relation to the Statement of Work outlined in the proposal.** No change in the original "Statement of Work" is requested at this time. At a later date, if data from preliminary studies warrant a change, the methods that we use to analyze *p53* gene sequence may be changed. In addition, since the primary focus of our study is breast CIS and not other pre-neoplastic histopathologies we are finding that atypical hyperplasias and other non-CIS pathologies are represented in only a limited number of the breast biopsies. The original "Statement of Work" is as follows:

## STATEMENT OF WORK.

### Technical Objectives:

**Task 1.** Identify and interview 100 African American and 426 white women with breast CIS in Los Angeles County during the first three years of the grant period. A previously tested epidemiologic questionnaire from the Women's CARE Study will be used to interview women entered in this CIS study.

**Task 2.** Identify and interview 490 African American and 490 white control women in Los Angeles County during the first three years of the grant period. These control women will be identified from the controls entered in the Women's CARE Study and interviewed as part of that study.

**Task 3.** Use an epidemiologic interview instrument to determine reproductive history including menarche, menopause, and pregnancy history, participation in physical activity / exercise, family history of breast cancer, race (white vs. black), body mass, and exposure to exogenous hormones (oral contraceptives and estrogen or combined estrogen/progestogen replacement therapy).

**Task 4.** Tissue blocks and slides will be obtained from the hospital laboratory where the diagnosis of breast CIS was made. The histopathology of each case will be reviewed and characterized.

**Task 5.** Gene amplification and expression of HER-2/*neu* and cyclin D1 will be evaluated in CIS, atypical hyperplasia, breast duct proliferative epithelium and normal epithelium using fluorescence *in situ* hybridization and immunohistochemistry.

**Task 6.** Alterations in *p53* expression and *p53* gene will be assessed in breast CIS, atypical hyperplasia, breast duct proliferative epithelium and normal epithelium using immunohistochemistry and a combination of SSCP and DNA sequencing.

**Task 7.** The frequency of alterations in the above oncogenes and p53 tumor suppressor gene will be compared with each other and with the epidemiologic data to assess the frequency of various associations. For example, how often is a history of birth control pill use associated with alterations of any of these genes in breast CIS cells?

**Task 8.** Use continued followup through the USC Cancer Surveillance Program and through annual contacts with patients and their physicians to determine how frequently women in the study develop recurrent or invasive breast cancer and assess how often these events are associated with particular genetic alterations in breast CIS.

**CONCLUSIONS:** The preliminary results obtained to date confirmed that ER, PR, p53 and HER-2/*neu* proteins are present in varying proportions in breast carcinoma *in situ*. The pilot study of p53 mutations in breast carcinoma *in situ* showed that p53 mutations and p53 overexpression were relatively common in intraductal breast carcinomas and are not observed in adjacent normal breast lobules or ducts. There was a highly significant correlation between p53 mutations and p53 overexpression in breast CIS.

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**APPENDICES:**

1. Lukas J, Niu N, Press MF. *p53* mutations in intraductal breast carcinomas. (in review), 1997.

***p53* Mutations in Intraductal Breast Carcinomas.**

by

Jason Lukas<sup>1</sup>, Ning Niu<sup>1</sup>, and Michael F. Press<sup>1,2</sup>

Department of Pathology<sup>1</sup> and the Norris Comprehensive Cancer Center<sup>2</sup>, University of Southern California School of Medicine, Los Angeles, California 90033

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Correspondence should be addressed to Michael Press, MD, PhD; Department of Pathology, Norris Comprehensive Cancer Center, NOR 5410, 1441 Eastlake Avenue, Los Angeles, CA, 90033; FAX 213-764-0122.

Keywords: intraductal breast carcinoma, breast carcinoma *in situ*, PCR, *p53* tumor suppressor gene, microdissection, immunohistochemistry, *p53* mutation.

## Summary

The *p53* tumor suppressor gene is altered in more than half of human cancers. Although *p53* mutations are common in invasive breast carcinoma, few have been identified in intraductal breast carcinomas (breast carcinoma *in situ*). The current study was undertaken to characterize *p53* in a cohort of breast carcinoma *in situ* (CIS) cases, both with and without invasive disease. Among breast CISs without invasive disease, 22% had *p53* mutations and 7% had DNA sequence alterations of unknown significance. *p53* overexpression was identified in 37% of intraductal breast carcinomas. *p53* mutations or alterations of unknown significance were concordant with *p53* overexpression in 87.5% of breast CIS cases lacking invasive disease. Analysis of breast CIS with invasive disease demonstrated *p53* mutations in 23% of cases and DNA alterations of unknown significance in 8%. Each carcinoma containing a *p53* mutation in the breast CIS component had the identical mutation in the invasive component of the same tumor suggesting a clonal relationship between the two tumor components. *p53* protein overexpression was identified in 38% of intraductal breast carcinomas with invasive disease and at approximately the same level in both components. Comparison of immunostaining and DNA sequence alterations showed a significant association between overexpression and mutations ( $p < 0.0001$ ). *p53* mutations and *p53* overexpression were relatively common in intraductal breast carcinomas but were not observed in adjacent normal breast lobules or ducts, indicating that *p53* alterations usually occur prior to invasion in breast carcinoma, as is observed for a number of other adult solid tumors.

## Introduction

Carcinoma of the breast is a multigenic disease which is generally thought to progress through a stepwise process that involves both morphologic and genotypic changes. It is thought that breast cancer develops from normal breast epithelium, progressing through epithelial hyperplasia without cytological atypia, hyperplasia with atypia, intraductal carcinoma or ductal carcinoma *in situ* (CIS), invasive cancer and, finally, metastatic disease<sup>1</sup>. Many genes are known

to be altered in invasive breast cancers; however, the specific time and phenotypic effect of these alterations during progression is not clear. Epidemiological studies have provided circumstantial evidence linking certain pre-invasive lesions to invasive breast cancer. Women diagnosed with ductal hyperplasia or atypical ductal hyperplasia have a two-fold or a 4 to 5-fold increased risk, respectively, of later invasive breast carcinoma relative to the unaffected population. A diagnosis of breast ductal CIS indicates an approximately 10-fold increased risk of future invasive breast carcinoma<sup>2</sup>. If the formation of invasive breast carcinomas is dependent on multiple genetic alterations, then morphologic phenotypes in breast pathology may coincide with these alterations.

Most investigations of p53 in breast cancer have been conducted in primary invasive breast carcinomas because of issues related to tumor availability. Approximately 22-58% of invasive breast cancers have p53 mutations or overexpression<sup>3-7</sup>. While the molecular genetic profile of p53 alterations has been reported in invasive breast carcinomas, most studies of breast CIS report only immunohistochemical analysis of p53 expression using paraffin embedded tissues<sup>8-20</sup>. Few studies have evaluated p53 mutations in breast CIS. Overall, only six p53 mutations have been described among 99 breast CIS cases (6%) screened by a variety of strategies<sup>21-24</sup>. The small number of mutations identified suggests that p53 mutations are infrequent in intraductal breast carcinomas. The higher rate of p53 mutations in invasive disease has suggested to some that p53 mutations are linked primarily to invasive breast carcinoma and occur relatively late in the disease process.

In the current study, p53 mutations were analyzed in exons 2 through 11 and p53 expression was analyzed by immunohistochemistry using frozen breast CIS specimens to more completely characterize p53 alterations in breast carcinoma *in situ*. The findings indicate that p53 is mutated in breast CIS as frequently as it is in invasive breast carcinoma and that the invasive carcinomas are clonally derived from breast CIS as indicated by maintenance of the same p53 sequence in intraductal and invasive disease from the same case.

## Results

**p53 mutations.** Fifteen of 40 cases initially screened by SSCP had altered mobility for at least one of the exons evaluated, exons 2 through 11 (Figure 1). Sequencing confirmed the presence of mutations in nine cases (Figure 2 and Table 1), demonstrated DNA sequence alterations of unknown significance in three cases and identified one DNA polymorphism.

Nine of 27 (33%) breast carcinoma *in situ* cases with no concurrent invasive carcinoma had altered mobility in one exon by SSCP, six (22%) mutations and two (7%) alterations of unknown significance were identified (Table 1). Seven of these eight cases of breast CIS with *p53* DNA sequence alterations had a comedocarcinoma histology and one had a papillary morphology. The DNA sequence alterations were distributed between exons 7 and 11. Four mutations, two Arg248Gln and two Arg249Gly, were found in exon 7. One Ala276Pro mutation was identified in exon 8 and one Gly325Ter mutation was identified in exon 9. Two identical base changes were noted in exon 11 after the termination codon (Table 1).

Three cases had sufficient DNA from benign epithelium for DNA sequence analysis. In the case with an exon 8 Ala276Pro mutation, DNA from 3 separate areas of benign and hyperplastic epithelium were sequenced and no *p53* mutation was observed. One case with an Arg249Gly mutation in CIS lacked this mutation in DNA from benign breast epithelium. However, one case with a DNA sequence alteration of unknown significance, a T->A base change after the termination site in exon 11, did contain the same sequence alteration in adjacent benign tissue as would be expected for a DNA polymorphism.

Among the 13 cases of breast CIS with invasive disease, five showed altered DNA conformations by SSCP; three (23%) of these were confirmed as having mutations, one had a DNA sequence alteration of unknown significance and one had a DNA polymorphism. Three DNA sequence changes were in exon 6, coding for a Leu194Ile mutation, a Tyr205Ser mutation, and a silent base change in Arg213 which is a known polymorphism<sup>25</sup>. The case with a Leu194Ile mutation showed wild-type *p53* in adjacent normal ductal epithelium. An Arg248Trp mutation was found in exon 7 (Table 1). In these cases with *p53* mutations, the same mutation



was noted in both the CIS component and in the invasive component. In cases without a *p53* mutation in the breast CIS component, no mutations were detected in the invasive component. No case had a *p53* mutation in the invasive component which was not also found in the concurrent breast CIS.

**p53 Protein Expression.** In the entire cohort, 15 of the 40 cases (37.5%) showed *p53* expression by immunohistochemical staining. *p53* protein was either exclusively or predominantly nuclear (Figure 3). Cytoplasmic staining without nuclear staining was not identified. The percentage of cells with nuclear staining for *p53* varied from 2% to 59%. In frozen tissue sections immunostaining is observed in nuclei of a low percentage of normal, proliferatively active tissues. Therefore, we have used 10% immunostained tumor cell nuclei as a value for separation of “normal” expression from “overexpression”<sup>26</sup>. Only two cases had less than 10% of tumor cell nuclei positively immunostained. One of these had a *p53* polymorphism (Table 1) and the other had wild-type *p53*.

*p53* overexpression was noted in 10 of 27 (37%) cases of breast CIS without invasion (Table 1). Average staining of tumor nuclei was 30% among breast CIS cases with overexpression. Among the 10 cases with *p53* overexpression, eight were classified as comedocarcinoma, one as papillary DCIS, and one as solid DCIS. Comedo DCIS with overexpression had a higher mean *p53* immunostaining than did breast CIS of other histologic types (34.3% for comedocarcinoma compared to 14.3% for other histologies). Six cases had sufficient benign epithelium for interpretation of immunostaining. Only two cases had positive immunostaining in benign hyperplasia, one of which had a *p53* mutation and overexpression in the breast CIS. The amount of breast hyperplasia tissue available from this case was, however, insufficient for DNA sequence analysis. The other case with breast hyperplasia without atypia demonstrated overexpression in the hyperplasia but did not show *p53* immunostaining in the CIS.

Five (38%) cases of CIS with invasive disease had *p53* overexpression. The percent of nuclei immunostained ranged from 14% to 65%. Average staining among these samples was 35.2%. Three cases with CIS components had comedocarcinoma histology, one had a

micropapillary histology, and one had a cribriform histology. Among these cases, there was complete concordance between the presence or absence of p53 immunostaining in the breast carcinoma *in situ* and the presence or absence of p53 immunostaining in the invasive breast carcinomas. Four cases with immunostaining in the CIS had no staining in the normal breast epithelium.

**Comparison of p53 mutations with overexpression.** Overall, 11 of the 40 cases (27.5%) had both p53 overexpression and either mutations or sequence alterations of undetermined significance, 24 had neither mutations nor overexpression, four had overexpression without mutations and one had a p53 mutation but lacked overexpression. The expression of P53 was highly correlated ( $p < 0.0001$ , Fisher's Exact test) with the presence or absence of mutations / sequence alterations (Table 2).

Among the 27 breast CIS cases without invasive disease, seven had both p53 mutations or alterations of unknown significance and overexpression, 16 had neither mutations nor overexpression, one had a p53 mutation but lacked overexpression, and three had overexpression but lacked p53 mutations. Among the 13 breast CIS cases with invasive carcinoma, four cases had both p53 mutations or DNA sequence alterations of unknown significance and overexpression, eight had neither p53 mutations nor overexpression, and one had p53 overexpression but lacked a p53 mutation (Table 1).

## Discussion

Breast carcinoma *in situ* (CIS) or intraductal breast carcinoma is one of the earliest recognizable forms of breast cancer. Breast CIS is confined to the lumen of breast ducts and lobules without penetration of the basement membrane and, therefore, without invasion of the breast stroma. However, cytological characteristics of intraductal tumor cells are essentially indistinguishable from the cytological characteristics of invasive breast carcinomas and intraductal carcinomas are considered to represent an early stage in a continuum of breast neoplasia. Circumstantial evidence supports this view. Breast CIS is associated with an increased risk of subsequent development of invasive breast carcinoma. Overexpression of HER-2/*neu* (*c-erb B-2*)

and other oncoproteins including p53 tumor suppressor protein product, known to be frequently overexpressed in invasive breast cancer, are also frequently overexpressed in breast CIS. Although overexpression of oncoproteins have been observed in breast CIS, these are immunohistochemical staining studies of archival, paraffin-embedded tissues which do not provide direct evidence of genetic alterations.

In previous reports 99 intraductal breast carcinoma cases have been preliminarily screened for p53 mutations using constant denaturant gel electrophoresis (CDGE)<sup>23</sup>, immunohistochemical staining<sup>21, 22</sup> and/or SSCP<sup>22, 24</sup>. Only six potential p53 gene mutations were confirmed by DNA sequence analysis in these studies of breast carcinoma *in situ*<sup>21-24</sup>. The p53 gene mutations include five missense mutations, an Arg202His<sup>24</sup>, a Met237Ile<sup>24</sup>, a Gly248Asp<sup>21</sup> and two Arg273His mutations<sup>22, 23</sup> and one frame-shift mutation, a single-base deletion in codon 304 which is predicted to result in the introduction of a premature termination site at codon 344<sup>24</sup> (Figure 4). The frequency of confirmed p53 mutations in the breast CIS cohorts screened was 3%, 4%, 7% and 11%<sup>21-24</sup>.

The p53 gene is reported to be mutated in approximately 22% of invasive breast cancers<sup>27, 28</sup>, while p53 overexpression is found in from 22% to 58% of cases<sup>3-7</sup>. This differential between reported frequencies for p53 mutations and p53 expression is similar to observations made here in breast CIS. In the present study, the frequency of p53 mutations in breast CIS lacking invasive carcinoma (22%) and the frequency of p53 mutations in breast CIS with invasive carcinoma (23%) was similar. The frequency of p53 overexpression in breast CIS lacking invasion (37%) was also similar to the frequency of p53 overexpression in breast CIS cases having invasion (38%). These results suggest that p53 mutations are relatively 'early' in the disease process occurring primarily in intraductal breast carcinomas.

In this cohort there was no evidence of an increase in p53 expression during progression from CIS to invasive disease within the same tumor (Table 1). Those breast tumors with both a CIS component and an invasive component with wild-type p53 in the CIS component by DNA

sequence analysis also had wild-type p53 in the invasive component. Furthermore, p53 mutations identified in a CIS component by DNA sequence analysis had the identical mutation detected in the invasive component of the tumor. These results provide evidence that *p53* mutations arise in preinvasive breast ductal CIS and that invasive carcinoma is clonally derived from CIS.

The estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor (EGFr) and HER-2/*neu* oncoprotein content had been assessed separately in these tumors using immunohistochemistry (Lukas and Press, unpublished observations). DNA ploidy was assessed using Feulgen staining and computerized image analysis (Becton Dickinson, San Jose, CA)<sup>29</sup>. Comparison of p53 mutations and overexpression with these tumor markers revealed no associations with ER, PR, EGF receptor or DNA ploidy. There was, however, a statistically significant association between high levels of HER-2/*neu* protein expression and p53 mutations ( $p=0.016$ ). If the DNA alterations after the termination codon in exon 11 are assessed as mutations, this association becomes stronger ( $p=0.005$ ). If all DNA sequence alterations of unknown significance are considered mutations, the association between high HER-2/*neu* expression and p53 mutation is still significant ( $p=0.026$ ). High HER-2/*neu* expression has been previously reported as showing a correlation with p53 expression in invasive breast carcinoma<sup>30</sup>.

The crystal structure analysis of p53 protein provides new insight regarding several important functional aspects of this molecule. The p53 core domain broadly consists of a sheet structure which supports loops and helices that interact with DNA. The support structure consists of two antiparallel  $\beta$  sheets which pack together to form a sandwich with a hydrophobic core. This sandwich supports the L1, L2 and L3 loops, as well as the H1 and H2 helices which interact with DNA. These loops and helices lack secondary structure, and thus stabilization is largely accomplished by the sharing of a zinc ion and several interactions between side chains or the amino acid backbone<sup>31-33</sup>. Mutations which effect DNA binding as well as mutations which effect the structure of the core domain as a whole can disable p53 function.

The *p53* mutations reported here can be divided into 3 groups. Mutations were found in sites which directly interact with DNA (Arg248Gln, Arg248Trp, Ala276Pro), in sites which preserve

*p53* structural integrity (Leu194Ile, Tyr205Ser, Arg249Gly) and in sites outside the core domain (Gly325Ter and alterations of unknown significance). Among the nine mutations, four interfere directly with *p53*/DNA binding, four interfere with internal stabilization of *p53* and one codes for a termination site in the 3' oligomerization domain. Three alterations of undetermined significance were also outside the core domain and are discussed separately.

Three mutations were at Arg248 and, therefore, interfere with *p53*/DNA binding. This codon, known to be critical to the anchoring of *p53* in the minor groove of DNA with 4 hydrogen bonds from 3 nitrogen atoms, is the most frequently mutated codon. In this cohort, Arg248 is mutated to Arg248Gln and Arg248Trp, neither having the number of nitrogen atoms necessary for bonding. Ala276 is important to *p53*/DNA binding as the nitrogen of alanine binds to the phosphate group of DNA. The Ala276Pro mutant does not have an nitrogen and will not bind DNA correctly. Each of these incorrect amino acids cannot form bonds necessary for *p53* stabilization and function.

Four mutations effect the ability of *p53* binding to DNA indirectly by altering the structural integrity of the *p53* molecule. An amino acid change, Leu194Ile at the junction of the L2 loop and the  $\beta$  sheet (Figure 4), probably indirectly destabilizes the Arg248 binding<sup>34</sup>. The Tyr205Ser mutation changes the central codon in the S6  $\beta$  strand. Here the  $\beta$  sheets are tightly packed together forming the hydrophobic core<sup>33</sup>. The loss of the tyrosine's hydrophobic phenolic side chain may alter the ability of these sheets to interact properly and, therefore, alter the protein structure. Arg249 is a frequently altered codon which links the L2 loop to the L3 loop with hydrogen bonds, stabilizing the Arg248 interaction in the minor groove. Two cases have an Arg249Gly mutation and this change would destabilize the L3 interaction in the minor groove. These mutations effect the overall structure of *p53*, and can have an impact on the ability of *p53* to bind DNA.

Gly325 is in the oligomerization domain, 3' to the DNA binding 'core' of *p53*. This domain extends from codon 319 to 360, consisting of a bend, a  $\beta$  strand, another short bend, and an  $\alpha$



helix, and is required for p53 protein to properly tetramerize<sup>38</sup>. A termination codon at Gly325, which corresponds to the first bend in the domain, would create a truncated mutant that would abolish proper oligomerization.

The Arg213 alteration does not code for an amino acid change. This codon is the most frequently altered non-missense codon<sup>35</sup> and is reported to be a polymorphic site<sup>25</sup>. Noncoding DNA sequence alterations have been reported at high frequency in sporadic breast tumors<sup>36</sup> and certain silent mutations at the wobble nucleotide in the fibroblast growth factor receptor 2 gene do appear have phenotypic effects<sup>37</sup>, possibly through the opening of a cryptic splice site.

A number of mutations effect codons outside of the core domain. A C-to-A alteration in the third intron is of undetermined significance as the change does not appear to open any cryptic splice sites but does have an increased percentage (14%) of nuclei immunostained for p53 protein.

Two identical T-to-A alterations, after the termination codon, were observed but are uncharacterized. Both cases had overexpression of p53 protein (47% and 40%), which suggested this DNA sequence alteration may effect the expression of p53, possibly in a manner similar to the control of  $\alpha$ -globin expression. Binding of a ribonucleoprotein complex to polycytosine runs in the 3' untranslated region of the  $\alpha$ -globin mRNA increase the halflife<sup>39</sup>. In *p53*, the site of the T -> A alteration is 5' - CCTCCCT/ACCCCC - 3', which is 91% cytosine and an exact match for the consensus binding site in  $\alpha$ -globin mRNA. Two or 3 base substitutions in the consensus binding site of  $\alpha$ -globin destabilizes the mRNA<sup>39</sup>, and it is possible that a similar binding protein may alter the half-life of p53 mRNA and increase translation of p53 protein in cases with single T -> A substitutions.

In conclusion, this study demonstrated *p53* mutations in breast carcinoma *in situ*. Identical *p53* mutations were identified in the CIS component and in the invasive component of several tumors. In addition, wild-type *p53* in the CIS component also corresponded with a wild-type *p53* sequence in the invasive component of the same tumor and not with the identification of a new *p53*

mutation in the invasive component. Because *p53* mutations and *p53* overexpression were common in breast carcinoma *in situ* and uncommon in adjacent normal breast lobules or ducts, and because no evidence of increasing *p53* mutations and *p53* immunostaining were observed with progression from breast CIS to invasive disease, it appears that mutations in *p53* often occur prior to invasion.

## Materials and Methods

**Tissues.** The use of human tissue in this study was reviewed and approved by the University of Southern California Institutional Research Committee. Forty frozen breast biopsy samples, stored at -186°C under liquid nitrogen, were used for these investigations. Thirteen cases had evidence of both invasive and *in situ* carcinoma and 27 cases had only carcinoma *in situ*. Twenty-four (60%) breast carcinoma *in situ*s were classified as comedocarcinoma, eight (20%) as cribriform ductal carcinoma *in situ* (DCIS), three (7.5%) as micropapillary DCIS, two (5%) as papillary DCIS, and three (7.5%) as solid DCIS. Frozen tissue sections, stained with hematoxylin and eosin, were used to confirm the histological composition of the specimens.

**Microdissection and DNA isolation.** The different tissue components of the breast specimens were separated according to histomorphologic phenotypes with the assistance of a dissecting microscope by microdissection of 10-20 serially cut, 10 µm thick, frozen tissue sections. Each section was fixed in 95% ethanol. The initial section was stained with hematoxylin-and-eosin and subsequent sections were stained with ethyl green. Microdissection was performed to separate benign breast epithelium, breast CIS and invasive carcinoma. Care was taken in order to minimize contamination of epithelial cells with stromal cells. After microdissection, each component was subjected to DNA extraction as described elsewhere<sup>40</sup>.

**p53 primer design.** The polymerase chain reaction (PCR) was used to amplify exons 2-11. Ten different sets of 19-25mer oligonucleotide primers were designed using the genomic sequence of *p53* (Genbank accession #X54156)<sup>41</sup>. Primers were designed to span each exon of

the *p53* open reading frame and sufficient bases of the intronic sequence to ensure the splice donor and splice acceptor sites were included<sup>26</sup>.

**Single-strand Conformational Polymorphism (SSCP) and DNA Sequence Analysis.** DNA sequence alterations in *p53* exons 2 through 11 were screened by altered mobility by the SSCP technique. Exons with altered mobility were analyzed for DNA sequence changes as described elsewhere using the CircumVent Thermal Cycle Sequencing Kit (New England Biolabs, Beverly, MA) or the ThermoSequenase Kit (Amersham, Arlington Heights, IL), according to the manufacturer's instructions<sup>40</sup>.

**p53 Immunohistochemistry.** The peroxidase anti-peroxidase technique was used to identify *p53* protein product in tissue sections. Frozen sections, 4 $\mu$ m thick, were incubated with anti-human *p53* mouse monoclonal antibody (DO-7 or Ab240, Dako Corporation, Carpinteria, CA or Zymed Laboratories, South San Francisco, CA, respectively) as previously described<sup>26</sup>. The percentage of positively immunostained tumor cell nuclei was determined from the number of nuclei containing immunoreaction product divided by the total number of nuclei, both immunostained and unstained. A minimum of 100 tumor cells were scored. Those breast tissues with *p53* immunostaining in at least 10% of the cell nuclei were considered to have *p53* overexpression, while those with less than 10% *p53* immunostained nuclei were considered to be within the normal range of *p53* expression<sup>26</sup>.

**Statistical Analyses.** The association between *P53* expression and *p53* mutations was evaluated using either Fisher's exact test. The Fisher Exact test was also used to evaluate potential associations between estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor (EGF) receptor and the presence of *p53* mutations. The Mantel-Haentzel test was used to evaluate potential associations between HER-2/*neu* receptor immunostaining and the presence of *p53* mutations and overexpression.

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